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(54) **Mutant B-type DNA polymerases exhibiting improved performance in PCR**

(57) Claimed are thermostable mutants of B-type DNA polymerases having a Y-GG/A amino acid motif between the N-terminal 3'-5'-exonuclease domain and

the C-terminal polymerase domain whereas the tyrosine of the Y-GG/A amino acid motif is mutated and whereas these mutant DNA polymerases are suitable for PCR.

	E	R	R	R	G(Y)	K	E	L	W	E	consensus											
T. aggregans	V	R	L	L	T	T	V	L	G	V	K	R	G	L	W	E	N					
T. litorea	V	R	L	L	T	T	V	L	G	V	K	R	G	L	W	E	N					
T. lummodans	V	R	L	L	T	T	V	L	G	V	K	R	G	L	W	E	N					
T. spec. 9N7	V	R	L	L	T	T	V	L	G	V	K	R	G	L	W	E	N					
T. gorgonarius	V	R	L	L	T	T	V	L	G	V	K	R	G	L	W	E	N					
P. spec. KOD	V	R	L	L	T	T	V	L	G	V	K	R	G	L	W	E	N					
P. abyssi	V	R	L	L	T	T	V	L	G	V	K	R	G	L	W	E	N					
P. furiosus	V	R	L	L	T	T	V	L	G	V	K	R	G	L	W	E	N					
P. horikoshii	V	R	L	L	T	T	V	L	G	V	K	R	G	L	W	E	N					
M. jannaschii	V	R	L	L	T	T	V	L	G	V	K	R	G	L	W	E	N					
M. caldococcus	V	R	L	L	T	T	V	L	G	V	K	R	G	L	W	E	N					
S. solfataricus	T	S	A	L	I	K	G	K	V	K	R	A	V	I	D	P	A	G	V	F	F	N
S. acidocaldarius	T	A	A	V	I	K	G	K	V	K	R	A	V	I	D	P	A	G	V	F	F	N
P. islandicum	T	A	A	V	I	K	G	K	V	K	R	A	V	I	D	P	A	G	V	F	F	N
P. oceanum	T	A	A	V	I	K	G	K	V	K	R	A	V	I	D	P	A	G	V	F	F	N
A. parvum	V	G	A	L	I	K	G	K	V	K	R	A	V	I	D	P	A	G	V	F	F	N
S. chaetoceros	T	A	A	V	I	K	G	K	V	K	R	A	V	I	D	P	A	G	V	F	F	N

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Figure 8

Description**FIELD OF THE INVENTION**

5 [0001] Subject of the invention is a thermostable mutant B-type DNA-polymerase having a Y-GG/A amino acid motif between the N-terminal 3'-5'-exonuclease domain and the C-terminal polymerase domain in the wild type form whereas amino acids of this motif are substituted in the mutant form of the DNA polymerase and whereas these mutant DNA polymerases are suitable for PCR reactions. Thermostable mutants according to the present invention exhibit better performance in PCR reactions compared to the wild type DNA polymerase. A further embodiment of the present invention is the use of these thermostable mutants of the B-type DNA polymerase for polymerase chain reactions (PCR) and other nucleic acid synthesizing reactions. Another subject of the present invention is a method of producing the inventive mutants, vectors and cell lines comprising genes encoding the inventive mutants.

BACKGROUND OF THE INVENTION

15 [0002] DNA-dependent DNA polymerases containing proof-reading activity have to coordinate two catalytic activities: the DNA polymerase activity and the exonuclease activity. For polymerase I type DNA polymerases (*E. coli* Pol I) as well as for B-type DNA polymerases, these catalytic activities are located on structurally distinct protein domains (Truniger, V., Lázaro, J., Salas, M. and Blanco, L. (1996) *EMBO J.*, 15(13), 3430-3441; Pisani, F. M., De Felice, M. and Rossi, M. (1998) *Biochemistry*, 37(42), 15005-15012). In B-type (eukaryotic-type) DNA polymerases, the coordination of the two catalytic activities was proposed to take place intramolecularly in the conserved motif Y-GG/A located between the N-terminal 3'-5' exonuclease and the C-terminal polymerization domain (Truniger, V., Lázaro, J., Salas, M. and Blanco, L. (1996) *EMBO J.*, 15(13), 3430-3441; Pisani, F. M., De Felice, M. and Rossi, M. (1998) *Biochemistry*, 37(42), 15005-15012). For the Klenow fragment of *E. coli* DNA polymerase it was described, that the editing can be an intermolecular or intramolecular process involving dissociation and reassociation of the DNA depending on the local context (Joyce, C. M. (1989) *JBC*, 264(18), 10858-10866).

Truniger et al. (Truniger, V., Lázaro, J., Salas, M. and Blanco, L. (1996) *EMBO J.*, 15(13), 3430-3441) demonstrated for the mesophile replicative DNA polymerase of bacteriophage ϕ 29 that mutations in the Y-GG/A motif can lead to phenotypes favoring either polymerisation or exonucleolysis compared to the wild type enzyme. They could show that this effect is related to altered (ss)DNA binding parameters and that the motif is important for the communication between the polymerase and exonuclease active site in a combination of structural and functional roles.

30 [0003] For the DNA polymerase of the thermophilic crenarchaeon *Sulfolobus solfataricus* (Sso) a region of 70 amino acids (region 1) involved in enzyme-DNA interaction was determined (Pisani, F. M., Manco, G., Carratore, V. and Rossi, M. (1996) *Biochemistry*, 35, 9158-9166). It is located in the connecting part between the exonuclease domain and the polymerase domain and contains the Y-GG/A motif. By mutational analysis of the amino acids in the Y-GG/A motif, it could be shown that the amino acids in this part of the enzyme determine the processivity of the proofreading function (Pisani, F. M., De Felice, M. and Rossi, M. (1998) *Biochemistry*, 37(42), 15005-15012). Based on the crystal structure of bacteriophage RB69 DNA polymerase, Truniger et al. proposed a direct interaction of the tyrosine with the phosphodiester bond between the two nucleotides preceding the one acting as template (Truniger, V., Blanco, L. and Salas, M. (1999) *J. Mol. Biol.*, 286, 57-69).

DESCRIPTION OF THE INVENTION

45 [0004] The subject of the present invention was to provide thermostable DNA polymerases exhibiting an improved performance in PCR. Especially, thermostable mutants of a B-type DNA polymerase are provided which exhibit improved PCR performance. The inventive mutants of the B-type DNA polymerase have mutations in the Y-GG/A amino acid motif. Preferred mutations refer to the position of the tyrosine in the Y-GG/A amino acid motif. Other mutations affecting the motif could also influence the performance of B-type DNA polymerases in PCR.

50 [0005] According to the present invention an improved performance of a DNA polymerase in PCR is defined as a performance that results in higher yields of PCR product, or the amplification of longer DNA targets. Additionally, improved PCR performance can be defined as improved fidelity during the amplification process.

[0006] Preferred mutant B-type DNA polymerases have mutations at the position of the tyrosine in the Y-GG/A amino acid motif. Preferred mutants of B-type DNA polymerases according to the present invention have phenylalanine, tryptophan or histidine at the position of the tyrosine. Other preferred mutants of B-type DNA polymerases according to the present invention have asparagine or serine at the position of the tyrosine. These mutant polymerases described here, in which the tyrosine of the Y-GG/A motif was substituted, exhibit an improved performance in PCR.

55 [0007] In a preferred embodiment the inventive mutant B-type DNA polymerase is a mutant of a B-type DNA polymerase obtainable from Euryarchaea, more preferably from *Thermococcus aggregans* (Tag). Especially preferred is a

mutant of a B-type DNA polymerase from *Tag* of about 94 kDa size with a temperature optimum of $\geq 80^\circ\text{C}$ and the ability to perform polymerase chain reactions.

[0008] The present invention is described in detail for the B-type DNA polymerase from *Thermococcus aggregans*, but the invention could also be applied to other B-type DNA polymerases. Preferably to those B-type DNA polymerases showing a high degree of homology ($\geq 80\%$) to the DNA polymerase from *Thermococcus aggregans*.

The B-type DNA polymerase from *Thermococcus aggregans* exhibits a high degree of amino acid sequence homology to B-type DNA polymerases of other *Thermococcus* species. The homology of the DNA polymerases was calculated using the program Blast 2 (Tatusova, T.A. and Madden, T.L. (1999) *FEMS Microbiol. Lett.* 174, 247 - 250). The homology of the B-type DNA polymerase from *Thermococcus aggregans* to the homologue enzymes from *Thermococcus* species is: 93% (*T. litoralis*), 87% (*T. gorgonarius*), 86% (*T. furiosus*) and 87% (*T. spec. 9N7*). The homology of the *Tag* DNA polymerase to polymerases from *Pyrococcus* species is: 86% (*P. abyssii*), 86% (*P. horikoshii*), 86% (*P. spec. KOD*) and 85% (*P. furiosus*). A lower homology is calculated to other B-type DNA polymerase from different euryarchaeota: 59% (*Methanococcus jannaschii*), 56% (*Methanococcus voltae*), 51% (*Methanobacterium thermoautotrophicum*) and 56% (*Archaeoglobus fulgidus*). To B-type DNA polymerases from crenarchaeota and bacteriophages the homology is found as follows: 46% (*Sulfolobus solfataricus*), 42% (*Sulfolobus acidocaldarius*), 41% (*Sulfurisphaera ohwakuensis*), 51% (*Aeropyrum pernix*), 40% (*Pyrodicticum occultum*), 43% (*Cenarchaeum symbiosum*), 38% (bacteriophage T4) and 39% (bacteriophage RB69).

[0009] As described above several mutations in the Y-GG/A motif were performed for the *Sulfolobus solfataricus* (Sso) and the $\phi 29$ DNA polymerases. The observed effects on polymerase activity (pol) and exonuclease activity (exo) of these mutations do not completely correspond to the effects obtained for the *Tag* DNA polymerase. Thus the effect of the mutations on the performance of the mutants in PCR was not predictable.

The mutant Y387F of the *Tag* DNA polymerase exhibits a higher pol/exo ratio compared to the wild type *Tag* DNA polymerase. Similar results were described for Sso and $\phi 29$ DNA polymerase. The mutant G389A displays the opposite effect than the corresponding mutant in $\phi 29$ DNA polymerase: while G \rightarrow A in *Tag* DNA polymerase almost knocks out polymerase activity, in $\phi 29$ DNA polymerase G \rightarrow A mutant this activity is clearly enhanced. For mutants of the Sso DNA polymerase a change of exonuclease processivity was described. Again, this was not observed for mutants of the B-type *Tag* DNA polymerases. Thus, a prediction of the effect of analogous mutants in the Y-GG/A motif could not be made.

[0010] In summary, although it has been described in the prior art that the Y-GG/A motif plays a role in the coordination of the DNA polymerase activity and the exonuclease activity, the observed changes of the pol/exo ratio of the prior art DNA polymerases do not strictly correlate to the changes observed for the inventive mutants of the *Tag* DNA polymerase. Furthermore, it has not been described that the Y-GG/A motif is important for the performance of B-type DNA polymerase in PCR. Additionally, there is no correlation between the changes of the pol/exo ratio and the improvement of the performance of DNA polymerases in PCR. For instance, the mutant Y387H does not exhibit a change of pol/exo ratio compared to the wild-type, but it exhibits improved performance in PCR. Furthermore, a significant enhancement of fidelity was observed for the mutants Y387N and Y387S of *Tag* DNA polymerase.

[0011] Results obtained for the mutants of *Tag* DNA polymerase are described in more detail below.

Enzymatic activities of wild type and mutant *Tag* DNA polymerases

[0012] The enzymatic activities of the wild type enzyme and the mutants of *Tag* DNA polymerase were determined and analyzed (Figure 1). The DNA polymerase activity was determined as described in Example 2. According to the effect of the mutations on the polymerase activity three groups of mutants were defined: i) mutants with enhanced DNA polymerase activity (mutant Y387F), ii) mutants having a similar or slightly reduced DNA polymerase activity compared to the wild type (mutants Y387W and Y387H) and iii) mutants with reduced DNA polymerase activity (mutants Y387N, Y387S, G389A).

The exonuclease activity was determined as described in Example 3. According to the effect of the mutations on the exonuclease activity two groups of mutants were defined: i) mutants with similar exonucleolytic activity as the wild type enzyme (mutants Y387F, Y387W, Y387H), ii) mutants with enhanced exonuclease activity (mutants Y387N, Y387S, G389A) in comparison to the wild type enzyme.

[0013] From the data obtained for polymerase activity and exonuclease activity the ratios of both activities (pol/exo) were calculated for the wild type enzyme and the mutants of *Tag* DNA polymerase (Figure 1). Three mutants showed a higher or similar pol/exo ratio as the wild type enzyme (mutants Y387F, Y387W, Y387H). Three mutants showed a clearly reduced pol/exo ratio in comparison to the wild type enzyme (mutants Y387N, Y387S, G389A).

PCR performance

[0014] Wild type and mutant enzymes were submitted to polymerase chain reactions on lambda DNA in a buffer optimized for this purpose. All mutants except for mutant G389A were able to perform PCR, but yielded different

amounts of product with a constant amount of enzyme (1 pmol). With increasing length of the DNA target, differences in performance of the enzyme were shown (Fig. 2). With 1 pmol of the mutants Y387S, Y387N and G389A no PCR product could be obtained for the amplification of a 3.3 kb fragment. 1 pmol of the wild type DNA polymerase could not amplify fragments of 5.0 kb length. The mutants Y387W, Y387F and Y387H were able to amplify a fragment of 7.5 kb length. As control *Taq* DNA polymerase, *Pwo* DNA polymerase and Expand™ High Fidelity PCR System (Roche Molecular Biochemicals) were used.

[0015] The differences in PCR performance were also shown by the amplification of a 2 kb fragment applying different elongation times in the PCR runs. Under these conditions, all enzymes tested except the mutant G389A were able to amplify a 2 kb fragment at an elongation time of 90 sec/cycle. The mutants Y387F, Y387W and Y387H were able to amplify the fragment at a reduced elongation time of 40 sec/cycle. The mutant Y387H was able to amplify the target in a elongation time of 30 sec/cycle (Fig. 3).

Exonuclease processivity

[0016] The exonuclease processivity of the enzymes was studied in an experiment based on the heparin trap method (Reddy, M. K., Weitzel, S. E. and von Hippel, P. H. (1992) *J. Biol. Chem.*, 267(20), 14157-14166; Pisani, F. M., De Felice, M. and Rossi, M. (1998) *Biochemistry*, 37(42), 15005-15012). A constant amount (1 pmol) of *Tag* DNA polymerase or its mutants was incubated for 4 minutes at 68 °C with a 5'-DIG-labelled 24mer oligonucleotide in the absence of nucleotides. In the absence of heparin, the oligonucleotide was continually degraded by the *Tag* enzymes (positive control, Fig. 4, lanes "-"). The function of the heparin trap method was demonstrated by addition of heparin and MnCl₂ before the binding of the enzyme (negative control, Figure 4, lanes B). Single turnover conditions (addition of heparin and MnCl₂ to start the reaction after the binding of enzyme) resulted in exonucleolytic degradation of the oligonucleotide by the *Tag* DNA polymerases (Fig. 4, lanes A). The enzymes showed differences in the exonucleolytic activity as shown by the different amounts of remaining oligonucleotide that was not degraded. However, for all enzymes tested the oligonucleotide was degraded to a similar extent (8 nt). This indicates a similar exonuclease processivity for the enzymes.

The *Thermococcus gorgonarius* DNA polymerase, which exhibits a strong exonuclease activity, was used as a positive control. It degraded the 24mer oligonucleotide in the absence of heparin to oligonucleotides of less than 15 bases length (Figure 4, lane "-"). Under single turnover conditions a strong degradation (11 nt) of the oligonucleotide is observed (Figure 4, lane "A").

Fidelity

[0017] The error rates in amplification were determined for the mutant enzymes and the wild type DNA polymerase. The PCR-based fidelity assay described by Frey and Suppmann (Frey, M. and Suppmann, B. (1995) *Biochemica*, 2, 34-35) was used. This method is based on the amplification, circulation and transformation of the pUC19 derivative pUCQ17, which contains a functional *lacI*^q allele (Barnes, W. M. (1994) *Proc. Natl. Acad. Sci. USA*, 91, 2216-2220). PCR-derived mutations in *lacI* result in a de-repression of the expression of *lacZα* and subsequent formation of a functional β-galactosidase enzyme, which can be detected on X-Gal indicator plates.

[0018] In five independent runs, a mean error rate of 5.0×10^{-6} was found for the wild type *Tag* DNA polymerase. This value is in between the mean error rates of 1.8×10^{-6} for Expand™ High Fidelity PCR System (Roche Molecular Biochemicals) and 1.3×10^{-5} for *Taq* DNA polymerase (Roche Molecular Biochemicals) determined in the corresponding experiments. For a better comparison of the data, we plotted the quotient of the error rate determined for *Taq* DNA polymerase divided by the error rates determined for the *Tag* DNA polymerase and its mutants. In the independent experiments the error rate for *Taq* DNA polymerase varied from 1.2 to 3.05×10^{-5} .

Figure 5 shows the quotients of the error rates of the wild type enzyme and mutants of *Tag* DNA polymerase. The error rates of the mutants showing improved PCR performance (Y387W, Y387F, Y387H) did not significantly differ from the values obtained for the wild type enzyme. The mutants with enhanced exonuclease activity (Y387N, Y387S) showed improved fidelity rates (Figure 5). For the mutants Y387N and Y387S mean error rates of 6.3×10^{-7} and 6.2×10^{-7} were determined.

[0019] In contrast to the φ29 DNA polymerase and the *Sso* DNA polymerase, the *Tag* DNA polymerase, is suited for PCR. The mutant enzymes (Y387F, Y387W, Y387H) with an aromatic amino acid in the position of the tyrosine showed a similar or only slightly enhanced DNA polymerase activity (mutants Y387F, Y387W, Y387H) but an improvement in PCR performance.

[0020] In the fidelity assay it was found that the mutants Y387F; Y387W and Y387H showed no significant change in their error rate. By contrast, the mutants Y387N or Y387S showed higher exonuclease activity and displayed an improved fidelity.

[0021] Subject of the present invention is also a method of producing the inventive B-type mutants comprising the

following steps: cloning and mutagenesis of the gene, followed by the expression and purification of the protein.

[0022] Subject of the present invention is a DNA encoding for a thermostable B-type DNA polymerase having a Y-GG/A amino acid motif between the N-terminal 3'-5' exonuclease domain and the C-terminal polymerase domain in the wild type enzyme whereas the tyrosine of this motif is substituted in the mutant enzyme of the polymerase and

whereas this mutant DNA polymerase is suitable for PCR.
[0023] Preferably, said DNA in wild type form is obtainable from Euryarchaea, more preferably from *Thermococcus aggregans* (*Tag*). Subject of the present invention is also a vector containing the inventive DNA. Suitable vectors are e.g. the following: pET14b/15b/16b/19b (Novagen); pRSET (Invitrogen); pTrcHis (Invitrogen); pHAT10/11/12 (Clontech); pPRO Tet.E/Lar.A (Clontech); pCALn/n-EK (Stratagene); pGEMEX-1/-2 (Promega).

[0024] Furthermore, subjects of the present invention are also cells comprising the above vector. Suitable cells are e.g. *E. coli* BL21, BL21(DE3), BL21(DE3)pLysS, BL21(DE3)pLysE, DH5 α -PRO, JM109 (DE3), TOP10 in combination with the vectors recommended by the suppliers. The gene may have to be subcloned and the protein purification procedure may have to be adapted in the case of different expression vectors.

A sample of the recombinant strain expressing *Tag* DNA polymerase was deposited with the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) Mascheroder Weg 1b, D-38124 Braunschweig (DSM No. 13224).

[0025] A further subject of the invention is the use of the inventive mutant enzymes for synthesizing nucleic acids e.g. in PCR reactions.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Figures:

Figure 1.

[0026] Table showing the relative polymerase activities (Pol) and 3'-5'-exonuclease activities (Exo) of *Tag* DNA polymerase and its mutants on double-stranded DNA.

Assays were carried out as described in example 2 and 3, respectively. The activities are expressed as percentage of the activity obtained for the wild-type *Tag* DNA polymerase.

Figure 2.

[0027] PCR with *Tag* DNA polymerase mutants.

Tag DNA polymerase mutants (1 pmol) were incubated in a 50 μ l total volume with 10 ng of lambda DNA as template and 30 pmoles of a primer set designed to yield the indicated fragment lengths, 200 μ M dNTPs and the suitable PCR buffer. Reactions were performed with 10 cycles of 10 sec 94 $^{\circ}$ C, 30 sec 57 $^{\circ}$ C and 3.0 min (A), 4.3 min (B) or 7.0 (C) min of elongation time at 72 $^{\circ}$ C followed by 20 cycles with elongation times increasing by 20 sec/cycle. After the PCR 5 μ l sample were submitted to electrophoresis on a 1% agarose gel. For the control reaction 2.5 U of *Taq* DNA polymerase, *Pwo* DNA polymerase or ExpandTM High Fidelity PCR System were used. The labeling of the lanes is described in the legend of figure 6.

Figure 3.

[0028] Time-dependent polymerase chain reaction.

1% agarose gels showing 2 kb PCR products from reactions performed with different elongation time (90 sec, 40 sec, 30 sec as indicated) to determine the minimal elongation time.

1 pmol of each *Tag* DNA polymerase mutant or wild type enzyme was added to a mix of 10 ng lambda DNA and primers designed to yield 2 kb DNA fragments. Labeling of the lanes is described in the legend of figure 6. For each mutant duplicate reactions were performed. Right lane of each gel *Pwo*: 2.5 U *Pyrococcus woesei* DNA polymerase (Roche Molecular Biochemicals) as control reaction. Left lane of each gel: Molecular weight marker VI (Roche Molecular Biochemicals). In the 40 sec reaction, mutant GA was omitted. In the 30 sec reaction, for the mutant YS only one reaction was run on the gel.

Figure 4.

[0029] 3'-5'-exonuclease processivity.

The *Tag* DNA polymerase mutants tested are indicated on top of the figure. *Tgo* DNA polymerase was used as a control reaction (incubation for 30 sec). Reactions for wild type and mutants of *Tag* DNA polymerase were performed for 4 minutes at 68 $^{\circ}$ C after preincubation for 1 minute at 68 $^{\circ}$ C. Lane "P" is the control reaction (24 mer 5'-DIG-labelled

primer without incubation), lane "-": reaction without heparin (positive control); lane "B": reaction with heparin and $MnCl_2$ added before addition of the enzyme (negative control); lane "A": heparin and $MnCl_2$ added after the enzyme (reaction under single turnover conditions).

5 Figure 5.

[0030] Fidelity of *Tag* DNA polymerase mutants.

The fidelity of *Tag* DNA polymerase and its mutants was expressed in relation to the fidelity of *Taq* DNA polymerase. A quotient of 1 means that the polymerase has the same error rate as *Taq* DNA polymerase (mean value 1.3×10^{-5}).
 10 Values >1 reflect the factor by which a polymerase shows less errors than *Taq* DNA polymerase. The bars correspond to mean values calculated from 2-5 independent experiments, error bars missing are smaller than 0.36. Abbreviations for enzymes are as indicated in legend to figure 6. As controls *Pyrococcus woesei* DNA polymerase (Roche Molecular Biochemicals) and Expand High Fidelity PCR System (Roche Molecular Biochemicals) were used ("Taq/Pwo" and "Taq/HiFi").

15

Figure 6.

[0031] SDS-PAGE gel analysis of purified mutant proteins.

1 μ g of each mutant was submitted to electrophoresis on an 10% SDS-PAGE gel. Left: MW, molecular weight marker;
 20 WT, *Thermococcus aggregans* wild type DNA polymerase; YF, YW, YS, YN, YH are the corresponding mutants with an exchange at the position of tyrosine 387 to phenylalanine, tryptophan, serine, asparagine, histidine, respectively. GA, mutation of glycine 389 to alanine in the gene of the *Thermococcus aggregans* DNA polymerase. All mutants showed the same chromatographic behaviour and solubility as the wild type enzyme.

25 Figure 7.

[0032] Qualitative exonuclease assay.

A DNA molecular weight marker was used as substrate to test the exonucleolytic activity (DNA molecular weight marker II (MW II), Roche Molecular Biochemicals). 1 μ g of MW II was incubated for 6 h at 65 °C with 1 pmol of each variant of *Tag* DNA polymerase in the presence (A) or absence (B) of 200 μ M dNTP. *Tag* mutants are named as explained in
 30 legend of figure 6. Exonucleolytic degradation take place only in the absence of deoxynucleotides. The qualitative ranking of the proteins in terms of exonuclease activity is GA > YN > YS > YH > YF = YW = WT.

Figure 8.

35

[0033] Consensus sequence motif for B-type DNA polymerases from the order of *Thermococcales* derived from a multiple alignment of amino acid sequences of euryarchaeal and crenarchaeal B-type DNA polymerases.

A region of 24 amino acids containing the Y-GG/A motif was analyzed with the ClustalW Software program (Higgins, EMBL Heidelberg, Germany). In addition to the amino acids conserved in all archaeal B-type DNA polymerases (like
 40 the Y-GG/A motif), a consensus sequence "E--RR-R----G(Y)-KE-EE--LWE-" can be defined. This sequence is found in the sequence of all DNA polymerases belonging to the order of the *Thermococcales* and coincides with a homology of >80% of the DNA polymerases.

The sequences of the crenarchaeal species *Sulfolobus solfataricus*, *Sulfolobus acidocaldarius*, *Pyrobaculum islandicum*, *Pyrodicticum occultum*, *Aeropyrum pernix*, *Sulfurisphaera ohwakuensis* and the sequences of several euryarchaeal species *Thermococcus* ("T."), *Pyrococcus* ("P.") and *Methanococcus* ("M.") were aligned.
 45

Figure 9.

[0034] DNA sequence and deduced amino acid sequence of recombinant wild type *Tag* DNA polymerase.

50 Three inteins found in the native gene (Acc. No. Y13030) were deleted by PCR (Niehaus, F., Frey, B., Antranikian, A. (1997) *Gene*, **204**, 153-158). Four mutations leading to amino acid exchanges were introduced during PCR. The amino acid exchanges (native→recombinant) are: L3F, A404T, S410C and L482H.

Example 1

55

Site-directed mutagenesis and expression of *Tag* DNA polymerase mutants

[0035] The cloning of the gene of *Tag* DNA Polymerase (polTY) was described earlier (Niehaus, F., Frey, B., Antran-

ikian, A. (1997) *Gene*, **204**, 153-158). Overexpression of *Tag* DNA Polymerase in *E. coli* was achieved by subcloning its encoding gene into the IPTG-inducible pET15b vector (Novagen) containing an N-terminal His-Tag for purification (the resulting plasmid was named pET15b-TagPol).

The mutants presented in this study were prepared in polymerase chain reactions using primers containing the desired mutations as a mismatch. The forward primer was universally "Kpn-fw", matching to a sequence about 100 bp upstream of the mutation site and contained a *KpnI* restriction site of the *polTy* gene. The reverse primers contained a *SnaBI* restriction site and additionally the desired mutation. The sequences of the oligonucleotides were as follows (mismatch sites for mutagenesis underlined):

SEQ. ID. NO: 1

Kpn-Fw 5'-GCAACCTTGTAGAGTAGAGTGGTACCTGTAAAGG-3';

SEQ. ID. NO: 2

TagY387F 5'-GCCTCTTTCCGGCTCTTTTACGTATCCTCCCAGGAAAGTAGTCC-3',

SEQ. ID. NO: 3

TagY387H 5'-GCCTCTTTCCGGCTCTTTTACGTATCCTCCCAGGTGAGTAGTCC-3',

SEQ. ID. NO: 4

TagY387N 5'-GCCTCTTTCCGGCTCTTTTACGTATCCTCCCAGGTTAGTAGTCC-3',

SEQ. ID. NO: 5

TagY387S 5'-GCCTCTTTCCGGCTCTTTTACGTATCCTCCCAGGGAAGTAGTCC-3',

SEQ. ID. NO: 6

TagY387W 5'-GCCTCTTTCCGGCTCTTTTACGTATCCTCCCAGCCAAGTAGTCC-3',

SEQ. ID. NO: 7

TagG389A 5'-GCCTCTTTCCGGCTCTTTTACGTATCCAGCCAGGTAAGTAGTCC-3'.

PCR reactions were carried out with Expand™ High Fidelity PCR System (Roche Molecular Biochemicals) using the following program: 2 min 94 °C, 30 cycles of 10 sec 94 °C, 30 sec 55 °C, 30 sec at 72 °C. The resulting 139 bp fragments were digested with the restriction enzymes *KpnI* and *SnaBI* yielding a 101 bp fragment that was ligated into pET15b-TagPol linearized with the restriction enzymes *KpnI* and *SnaBI*. The cloned DNA fragments were sequenced to confirm the presence of the desired mutations.

[0036] For protein expression, *E. coli* BL21 (DE3) cells were transformed with the expression vector pET15b-TagPol. Three to five colonies were inoculated in 15 ml of LB medium supplemented with 100 µg Ampicillin per ml and grown to OD_{600nm} 0.3. An aliquot (10 ml) of the preculture was used to inoculate 500 ml of LB medium and incubated while shaking at 37°C. At OD_{600nm} = 0.6 expression was induced by addition of IPTG (final concentration: 1 mM). After incubation for 3 hours cells were harvested by centrifugation and suspended in 50 mM Tris-HCl/pH 7.5, 10 mM KCl, 0.5 mM EDTA, 4 mM MgCl₂, 5 mM DTT. Cells were sonicated on ice and the crude extract was heated for 15 min to 80 °C. Cell debris was removed by centrifugation (30 min, 30 000 x g at 4°C).

The supernatant was applied to a Blue Sepharose 3G-A column (Pharmacia) equilibrated with buffer A (50 mM Tris-HCl/ pH 7.5, 10 mM KCl, 4 mM Mg Cl₂). The protein was eluted with a gradient of 0.01-1.5 M KCl. Active fractions were pooled and dialyzed against 20 mM Tris-HCl /pH 7.9, 5 mM imidazole, 500 mM NaCl. The sample was applied to a Ni-chelate column (Novagene) equilibrated in the same buffer and eluted with a gradient of 0.005 - 1 M imidazole. Active fractions were pooled and dialyzed against storage buffer (50 mM Tris-HCl/pH 7.5, 100 mM KCl, 0.5 mM EDTA, 5 mM DTT, 50% glycerol). The enzymes were pure as shown by SDS gel electrophoresis (Figure 6).

Example 2**DNA polymerase assay**

5 [0037] The DNA polymerase activity was determined by measuring the incorporation of α -(32 P)dCTP in a DNA substrate. The test mix (50 μ l) contained 5 μ l 10x *Tag* reaction buffer (100 mM Tris-HCl /pH 8.9, 750 mM KCl, 15 MgCl₂, 100 mM CHAPS), 200 μ M of each dATP, dGTP, dTTP, 100 μ M dCTP, 1 mCi α -(32 P)dCTP, 1 μ g of M13mp9 ssDNA annealed with 0.3 μ g M13 primer. Assays were performed with 2 and 3 μ l of enzyme in three different dilutions (final amount of enzyme 2.5 to 15 fmoles) yielding six reactions to calculate a mean value. As a reference *Pwo* DNA polymerase was used. The DNA/primer mix was prepared by heating 277.2 μ g M13mp9 ssDNA (Roche Molecular Biochemicals) and 156 μ g M13 sequencing primer (17mer forward primer, Roche Molecular Biochemicals) for 30 minutes to 55 °C and then cooling it for 30 minutes to room temperature.

10 [0038] Assay reactions were incubated for 30 minutes at 65 °C, stopped on ice by addition of 500 μ l of 10% TCA (4 °C) and kept on ice for another 10 minutes. Samples were filtered over GFC-filter (Whatman), filters washed three times with 5% TCA, dried and submitted to β -counting in 2 ml of scintillation fluid. One unit is defined as the amount of enzyme necessary to incorporate 10 nM dNTP into acid insoluble material at 65 °C in 30 minutes.

Example 3**Exonuclease Assays****Activity Assay**

25 [0039] 3 μ l (300 ng) of enzyme (approximately 5 Units of polymerase activity) were incubated with 5 pg of 3H-labelled calf thymus DNA for 4 hours at 65 °C in a buffer containing 10 mM Tris-HCl/pH 8.9, 75 mM KCl, 1.5 MgCl₂, 10 mM CHAPS. Radioactivity liberated from calf thymus DNA was measured in a scintillation counter.

The assay used does not discriminate between the 3'-5' exonuclease activity and the 5'-3' exonuclease activity. 5'-3' exonuclease activity has not been detected phenotypically or genotypically in B-type polymerases of *Thermococcales* (Perler, F. B., Kumar, S. and Kong, H. (1996) *Adv. Prot. Chem.*, **48**, 377-435). Thus the values obtained can be regarded as 3'-5'-exonuclease activity.

30 [0040] In another assay, 1 μ g of molecular weight marker II (Roche Molecular Biochemicals) were incubated in same buffer as above with 5 U of the indicated protein with or without 200 μ M dNTP in a final volume of 50 μ l for 6 hours at 65 °C. The reaction products were separated by electrophoresis on 1% agarose gels.

3'-5' Exonuclease processivity assay

35 [0041] The previously described heparin trap method was used (Reddy, M. K., Weitzel, S. E. and von Hippel, P. H. (1992) *J. Biol. Chem.*, **267**(20), 14157-14166; Pisani, F. M., De Felice, M. and Rossi, M. (1998) *Biochemistry*, **37**(42), 15005-15012). A reaction mix (10 μ l) containing 10 mM Tris-HCl/pH 8.9, 75 mM KCl, 10 mM CHAPS and 0.5 pmoles of a 5'-DIG-labeled 24mer oligonucleotide was prewarmed for 1 minute at 68 °C in a thermocycler. Unless otherwise noted, 1 pmol of the enzyme was preincubated for 1 minute at 68 °C with the substrate. The reaction was started by addition of MnCl₂ (final concentration: 4 mM) and heparin (final concentration: 1 mg/ml) to ensure single turnover conditions. After incubation for 4 minutes, the reaction was stopped by the addition of 5 μ l of formamide buffer (80% formamide, 10 mM EDTA, 1 mg/ml bromophenol blue, 1 mg/ml xylene xyanol). The efficiency of the heparin trap was checked in a control reaction by adding heparin and MnCl₂ prior to the addition of the enzyme. The samples were denatured for 3 minutes at 90 °C and subjected to denaturing gel electrophoresis on a 17.5 % polyacrylamide/8 M urea gel. Gels were blotted to a positively charged nylon membrane (Roche Molecular Biochemicals) and the blots developed with CPD-Star (Roche Molecular Biochemicals) according to the manufacturers instructions.

Example 4**lacI-based PCR fidelity assay**

50 [0042] We used the *lacI*-based PCR fidelity assay described by Frey and Suppmann (Frey, M. and Suppmann, B. (1995) *Biochemica*, **2**, 34-35). This method is based on the amplification, circularization and transformation of the pUC19 derivative pUCQ17, which contains a functional *lacI*^q allele (Barnes, W. M. (1994) *Proc. Natl. Acad. Sci. USA*, **91**, 2216-2220). PCR-derived mutations in *lacI* result in a de-repression of the expression of *lacZ α* and subsequent formation of a functional β -galactosidase enzyme, which can be easily detected on X-Gal indicator plates.

[0043] The truncated *lacI* gene of pUC19 was substituted by a functional copy of *lacI*^q. A 178 bp *Pvu II*-*Afl III* fragment was replaced by a 1121 bp DNA fragment encoding *lacI*^q. The α -complementing *E. coli* strain DH5 α , once transformed with the resulting plasmid pUCIQ17 (3632 bp), produces white (LACI⁺) colonies on LB plates containing ampicillin (100 μ g/ml) and X-Gal (0,004% w/v). For the PCR, pUCIQ17 was linearized by digestion with *Dra II* and used as a template in an amount of 1 or 10 ng. Both primers have *Cla I* cleavage sites at their 5' ends. Oligonucleotide Cla33 (34mer, 24 matches: SEQ. ID. NO: 8: 5'-AGC TTA TCG ATG GCA CTT TTC GGG GAA ATG TGC G-3') and Oligonucleotide Cla55 (36mer, 26 matches: SEQ. ID. NO: 9: 5'- AGC TTA TCG ATA AGC GGA TGC CGG GAG CAG ACA AGC-3') resulted in a PCR product of 3493 bp. The reactions were performed with 1 or 5 pmol of protein in the *Tag* polymerase PCR buffer described below or for the control reactions in the manufacturers PCR buffers with 2.5 U of enzyme. The cycle conditions were 10 sec denaturation at 94 °C, 30 sec annealing at 57 °C and 4 min elongation at 72 °C for 18, 24 or 30 cycles depending on the enzyme.

After PCR, the yield of amplification product was determined at (OD_{260nm} or in agarose gel) and the DNA fragments submitted to phenol/chloroform extraction to eliminate any protein. After digestion with *ClaI*, the DNA fragments were purified from a preparative agarose gel. Ligation reactions were carried out with the Rapid Ligation Kit (Roche Molecular Biochemicals), the reactions contained 30 ng DNA. The resulting circular plasmids were transformed in *E. coli* DH5 α as described by Hanahan (Hanahan, D. (1983) *J. Mol. Biol.*, 166, 557-580) and plated on LB Amp/X-Gal plates described above. After incubation overnight at 37 °C, blue and white colonies were counted. The error rate (f) per bp was calculated with a rearranged equation published by Keohavong and Thilly (Keohavong, P. and Thilly, W. G. (1989) *Proc. Natl. Acad.*

Sci. USA, 86, 9253-9257): $f = -\ln F / d \times b$ bp.

[0044] Where F is the fraction of white colonies (white colonies/ total colonies); d is the number of DNA duplications: 2^d = output DNA/input DNA and b is the effective target size (1080 bp) of the *lacI* gene. There are 349 phenotypically identified (by colour screening) single-base substitutions (non-sense and mis-sense) at 179 codons (approximately 50% of the coding region) within the *lacI* gene. (Provost, G. S., Kretz, P. L., Hamner, R. T., Matthews, C. D., Rogers, B. J., Lundberg, K. S., Dyaico, M. J. and Short, J. M. (1993) *Mut. research*, 288, 133-149). Frameshift errors which may occur at every position in the 1080 bp open reading frame of *lacI*, are not taken into account because little information is available for the specific polymerases used in PCR systems except for *Taq* DNA polymerase.

Example 5

Polymerase Chain Reactions

[0045] PCR was performed in a buffer optimized for *Tag* DNA polymerase and its mutants: 10 mM Tris-HCl/pH 8.9, 75 mM KCl, 1.5 MgCl₂, 10 mM CHAPS, 200 μ M dNTP. 10 ng of λ DNA were used as a template and 30 pmol of each primer (20 bp, designed to yield the products of the desired length):

Lambda 1, universal forward primer: 5'-GAT GAG TTC GTG TCC GTA CAA CA-3', SEQ. ID. NO: 10,

Lambda 3.3: 5'-CTC ATC AGC AGA TCA TCT TCA GG-3', SEQ. ID. NO: 11,

Lambda 8: 5'-ACT CCA GCG TCT CAT CTT TAT GC-3', SEQ. ID. NO: 12,

Lambda 9: 5'-GAT GGT GAT CCT CTC TCG TTT GC-3', SEQ. ID. NO: 13.

Lambda 3.3, 8 and 9 were used as reverse primers for the amplification of 3.3 kb, 5 kb and 7.5 kb fragments respectively. Template, primers and nucleotides were prepared in mix 1 in a volume of 25 μ l. Then 25 μ l of mix 2 containing the buffer and enzyme (1 pmol *Tag* wild type or mutant; or 2.5 U control enzyme) were added. All reactions were prepared in duplicate. The amplification was performed in a 2400 GeneAmp thermocycler (Perkin Elmer). The cycle conditions were: 2 min at 94 °C, 10 cycles with 10 sec denaturation at 94 °C, 30 sec annealing at 58 °C and elongation at 72 °C.

[0046] Elongation times depended on the length of the product (3 min for 3.3 kb, 4.3 min for 5 kb and 7 min for 7.5 kb). Another 20 cycles were performed with increasing the elongation times by 20 sec/cycle. The reaction was finished by 7 minutes at 72 °C. The tubes were kept at 4°C until separation by electrophoresis on 1% agarose gels.

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[0047] The improvement of the PCR performance of the mutants was also studied in a "time-dependent PCR". A 2 kb fragment was amplified from lambda DNA as described above. In these studies the elongation time of the PCR was stepwise reduced (90 sec, 40 sec, 30 sec) to determine for each enzyme the minimal elongation time that was sufficient to amplify the 2 kb fragment. The following primers were used:

Lambda 1, universal forward primer: 5'-GAT GAG TTC GTG TCC GTA CAA CA-3', SEQ. ID. NO: 14,
Lambda 6, reverse primer: 5'-CTT CAT CAT CGA GAT AGC TGT CG-3', SEQ. ID. NO: 15.

The temperature profile was as described above. The elongation times were kept constant over 30 cycles.

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25	gcc gaa agc gtt acc gcg tgg gga agg cac tac ata gaa atg acc ata	1584
	Ala Glu Ser Val Thr Ala Trp Gly Arg His Tyr Ile Glu Met Thr Ile	
	515 520 525	
30	aaa gag ata gag gag aaa ttt gga ttt aag gtg cta tat gcc gac act	1632
	Lys Glu Ile Glu Glu Lys Phe Gly Phe Lys Val Leu Tyr Ala Asp Thr	
	530 535 540	
35	gat ggt ttt tac gcc aca ata ccg gga gaa aaa cct gaa aca atc aaa	1680
	Asp Gly Phe Tyr Ala Thr Ile Pro Gly Glu Lys Pro Glu Thr Ile Lys	
	545 550 555 560	
40	aag aaa gct aag gaa ttc tta aaa tac ata aac tcc aaa ctt ccc ggt	1728
	Lys Lys Ala Lys Glu Phe Leu Lys Tyr Ile Asn Ser Lys Leu Pro Gly	
	565 570 575	
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	Leu Leu Glu Leu Glu Tyr Glu Gly Phe Tyr Leu Arg Gly Phe Phe Val	
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	Ala Lys Lys Arg Tyr Ala Val Ile Asp Glu Glu Gly Arg Ile Thr Thr	
	595 600 605	
55	agg ggt ctg gaa gtt gta agg agg gac tgg agc gaa ata gcc aaa gag	1872
	Arg Gly Leu Glu Val Val Arg Arg Asp Trp Ser Glu Ile Ala Lys Glu	
	610 615 620	
60	acc cag gct aaa gtc ttg gag gca ata ctt aaa gaa gat agt gtc gaa	1920
	Thr Gln Ala Lys Val Leu Glu Ala Ile Leu Lys Glu Asp Ser Val Glu	
	625 630 635 640	

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5 aaa gct gtg gaa atc gtt aag gac gtt gtt gag gag ata gca aaa tac 1968
 Lys Ala Val Glu Ile Val Lys Asp Val Val Glu Glu Ile Ala Lys Tyr
 645 650 655

 caa gtc ccg ctt gaa aag ctt gtt atc cac gag cag att acc aag gat 2016
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 660 665 670

 10 cta agt gaa tac aaa gcc att ggg cct cat gta gca ata gca aag agg 2064
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 675 680 685

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 15 Leu Ala Ala Lys Gly Ile Lys Val Arg Pro Gly Thr Ile Ile Ser Tyr
 690 695 700

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 705 710 715 720

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 725 730 735

 25 gaa aac caa gtt ctg ccg gcg gtg ctt agg atc ctt gaa gcc ttc ggc 2256
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 gac gcg tgg ctt aag aag tag 2325
 35 Asp Ala Trp Leu Lys Lys
 770 775

 40 <210> 34
 <211> 775
 <212> PRT
 <213> T. aggregans

 <400> 34
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 1 5 10 15

 Arg Ile Phe Lys Lys Glu Asn Gly Glu Phe Lys Ile Glu Leu Asp Pro
 20 25 30

 50 His Phe Gln Pro Tyr Ile Tyr Ala Leu Leu Lys Asp Asp Ser Ala Ile
 35 40 45

 55

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	Asp	Glu	Ile	Lys	Ala	Ile	Lys	Gly	Glu	Arg	His	Gly	Lys	Ile	Val	Arg	
	50						55					60					
5	Val	Val	Asp	Ala	Val	Lys	Val	Lys	Lys	Lys	Phe	Leu	Gly	Arg	Asp	Val	
	65					70					75					80	
	Glu	Val	Trp	Lys	Leu	Ile	Phe	Glu	His	Pro	Gln	Asp	Val	Pro	Ala	Leu	
					85					90					95		
10	Arg	Gly	Lys	Ile	Arg	Glu	His	Pro	Ala	Val	Ile	Asp	Ile	Tyr	Glu	Tyr	
				100					105					110			
	Asp	Ile	Pro	Phe	Ala	Lys	Arg	Tyr	Leu	Ile	Asp	Lys	Gly	Leu	Ile	Pro	
15			115					120					125				
	Met	Glu	Gly	Asp	Glu	Glu	Leu	Lys	Leu	Met	Ala	Phe	Asp	Ile	Glu	Thr	
	130						135					140					
20	Phe	Tyr	His	Glu	Gly	Asp	Glu	Phe	Gly	Lys	Gly	Glu	Ile	Ile	Met	Ile	
	145					150					155					160	
	Ser	Tyr	Ala	Asp	Glu	Glu	Glu	Ala	Arg	Val	Ile	Thr	Trp	Lys	Asn	Ile	
				165					170						175		
25	Asp	Leu	Pro	Tyr	Val	Asp	Val	Val	Ser	Asn	Glu	Arg	Glu	Met	Ile	Lys	
				180					185					190			
	Arg	Phe	Val	Gln	Ile	Val	Arg	Glu	Lys	Asp	Pro	Asp	Val	Leu	Ile	Thr	
30			195					200					205				
	Tyr	Asn	Gly	Asp	Asn	Phe	Asp	Leu	Pro	Tyr	Leu	Ile	Lys	Arg	Ala	Glu	
	210						215					220					
35	Lys	Leu	Gly	Val	Thr	Leu	Leu	Leu	Gly	Arg	Asp	Lys	Glu	His	Pro	Glu	
	225					230					235					240	
	Pro	Lys	Ile	His	Arg	Met	Gly	Asp	Ser	Phe	Ala	Val	Glu	Ile	Lys	Gly	
				245					250						255		
40	Arg	Ile	His	Phe	Asp	Leu	Phe	Pro	Val	Val	Arg	Arg	Thr	Ile	Asn	Leu	
				260					265					270			
	Pro	Thr	Tyr	Thr	Leu	Glu	Ala	Val	Tyr	Glu	Ala	Val	Leu	Gly	Lys	Thr	
45			275					280					285				
	Lys	Ser	Lys	Leu	Gly	Ala	Glu	Glu	Ile	Ala	Ala	Ile	Trp	Glu	Thr	Glu	
	290						295					300					
50	Glu	Ser	Met	Lys	Lys	Leu	Ala	Gln	Tyr	Ser	Met	Glu	Asp	Ala	Arg	Ala	
	305					310					315					320	
	Thr	Tyr	Glu	Leu	Gly	Lys	Glu	Phe	Phe	Pro	Met	Glu	Ala	Glu	Leu	Ala	
				325						330					335		
55																	

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Lys Leu Ile Gly Gln Ser Val Trp Asp Val Ser Arg Ser Ser Thr Gly
340 345 350

5 Asn Leu Val Glu Trp Tyr Leu Leu Arg Val Ala Tyr Glu Arg Asn Glu
355 360 365

Leu Ala Pro Asn Lys Pro Asp Glu Glu Glu Tyr Arg Arg Arg Leu Arg
370 375 380

10 Thr Thr Tyr Leu Gly Gly Tyr Val Lys Glu Pro Glu Arg Gly Leu Trp
385 390 395 400

Glu Asn Ile Thr Tyr Leu Asp Phe Arg Cys Leu Tyr Pro Ser Ile Ile
15 405 410 415

Val Thr His Asn Val Ser Pro Asp Thr Leu Glu Arg Glu Gly Cys Lys
420 425 430

20 Asn Tyr Asp Val Ala Pro Ile Val Gly Tyr Lys Phe Cys Lys Asp Phe
435 440 445

Pro Gly Phe Ile Pro Ser Ile Leu Gly Glu Leu Ile Thr Met Arg Gln
450 455 460

25 Glu Ile Lys Lys Lys Met Lys Ala Thr Ile Asp Pro Ile Glu Lys Lys
465 470 475 480

Met Leu Asp Tyr Arg Gln Arg Ala Val Lys Leu His Ala Asn Ser Tyr
30 485 490 495

Tyr Gly Tyr Met Gly Tyr Pro Lys Ala Arg Trp Tyr Ser Lys Glu Cys
500 505 510

35 Ala Glu Ser Val Thr Ala Trp Gly Arg His Tyr Ile Glu Met Thr Ile
515 520 525

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530 535 540

40 Asp Gly Phe Tyr Ala Thr Ile Pro Gly Glu Lys Pro Glu Thr Ile Lys
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Lys Lys Ala Lys Glu Phe Leu Lys Tyr Ile Asn Ser Lys Leu Pro Gly
565 570 575

45 Leu Leu Glu Leu Glu Tyr Glu Gly Phe Tyr Leu Arg Gly Phe Phe Val
580 585 590

Ala Lys Lys Arg Tyr Ala Val Ile Asp Glu Glu Gly Arg Ile Thr Thr
595 600 605

50 Arg Gly Leu Glu Val Val Arg Arg Asp Trp Ser Glu Ile Ala Lys Glu
610 615 620

55

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Thr Gln Ala Lys Val Leu Glu Ala Ile Leu Lys Glu Asp Ser Val Glu
 625 630 635 640
 5 Lys Ala Val Glu Ile Val Lys Asp Val Val Glu Glu Ile Ala Lys Tyr
 645 650 655
 Gln Val Pro Leu Glu Lys Leu Val Ile His Glu Gln Ile Thr Lys Asp
 660 665 670
 10 Leu Ser Glu Tyr Lys Ala Ile Gly Pro His Val Ala Ile Ala Lys Arg
 675 680 685
 Leu Ala Ala Lys Gly Ile Lys Val Arg Pro Gly Thr Ile Ile Ser Tyr
 690 695 700
 15 Ile Val Leu Arg Gly Ser Gly Lys Ile Ser Asp Arg Val Ile Leu Leu
 705 710 715 720
 20 Ser Glu Tyr Asp Pro Lys Lys His Lys Tyr Asp Pro Asp Tyr Tyr Ile
 725 730 735
 Glu Asn Gln Val Leu Pro Ala Val Leu Arg Ile Leu Glu Ala Phe Gly
 740 745 750
 25 Tyr Arg Lys Glu Asp Leu Lys Tyr Gln Ser Ser Lys Gln Val Gly Leu
 755 760 765
 30 Asp Ala Trp Leu Lys Lys
 770 775

35 Claims

1. A thermostable mutant B-type DNA polymerase having a Y-GG/A amino acid motif between the N-terminal 3'-5'-exonuclease domain and the C-terminal polymerase domain in the wild-type form of the polymerase whereas preferably the tyrosine of this motif is substituted in the mutant form of the polymerase and whereas this mutant DNA polymerase is suitable for polymerase chain reactions.
2. A mutant B-type DNA polymerase according to claim 1 having an amino acid with an aromatic side chain at the position of the tyrosine.
3. A mutant thermostable B-type DNA polymerase according to claim 1 having a Y→F, Y→W or Y→H mutation.
4. A mutant B-type DNA polymerase according to claim 1 having an amino acid with an hydrophilic side chain at the position of the tyrosine.
5. A mutant thermostable B-type DNA polymerase according to claim 1 having a Y→N or Y→S mutation.
6. A mutant thermostable B-type DNA polymerase according to claims 1-5 whereas the wild type form is obtainable from Euryarchaea.
7. A mutant thermostable B-type DNA polymerase according to claims 1-6 whereas the wild type form is obtainable from *Thermococcus aggregans*.
8. A mutant of a thermostable B-type DNA polymerase according to claims 1-6 whereas the amino acid sequence

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of the wild type form is $\geq 80\%$ homologue to the amino acid sequence of wild type *Tag* DNA polymerase.

9. A DNA encoding a thermostable mutant DNA polymerase according to claims 1-8.

5 10. A vector containing a DNA according to claim 9.

11. A transformed host cell comprising a vector according to claim 10.

10 12. A process for obtaining a polymerase according to claim 1-8 comprising the steps of cloning and mutagenesis of the gene, followed by the expression and purification of the protein.

13. Use of a polymerase according to claim 1-8 for synthesizing nucleic acids.

15 14. Use of a polymerase according to claims 1-8 for PCR reactions.

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Figure 1

Pol activity and Exo activity in %

	WT	Y387F	Y387W	Y387H	Y387N	Y387S	G389A
Pol	100	160	92	93,6	6,4	17,8	10,7
Exo	100	90	71	98	205	187	236
Pol/Exo	1	1,77	1,29	0,96	0,03	0,09	0,04

Figure 2

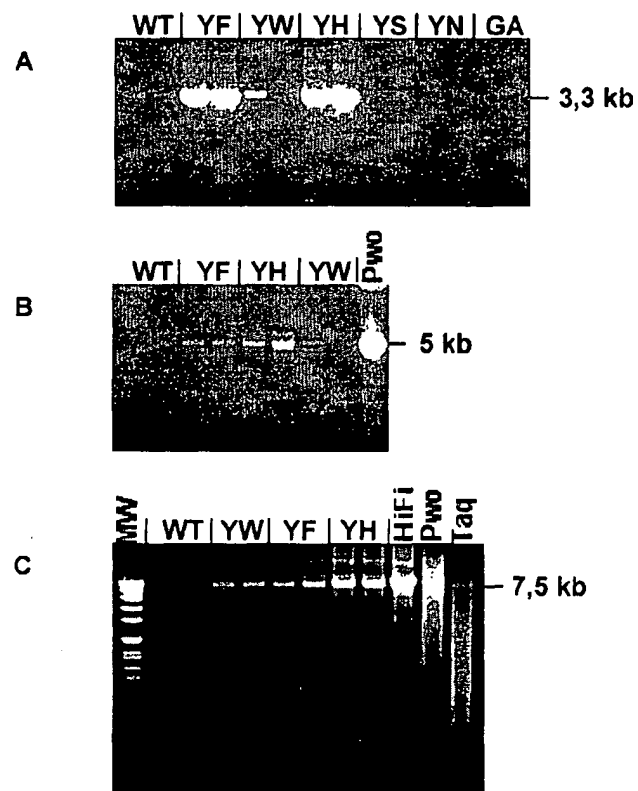


Figure 3

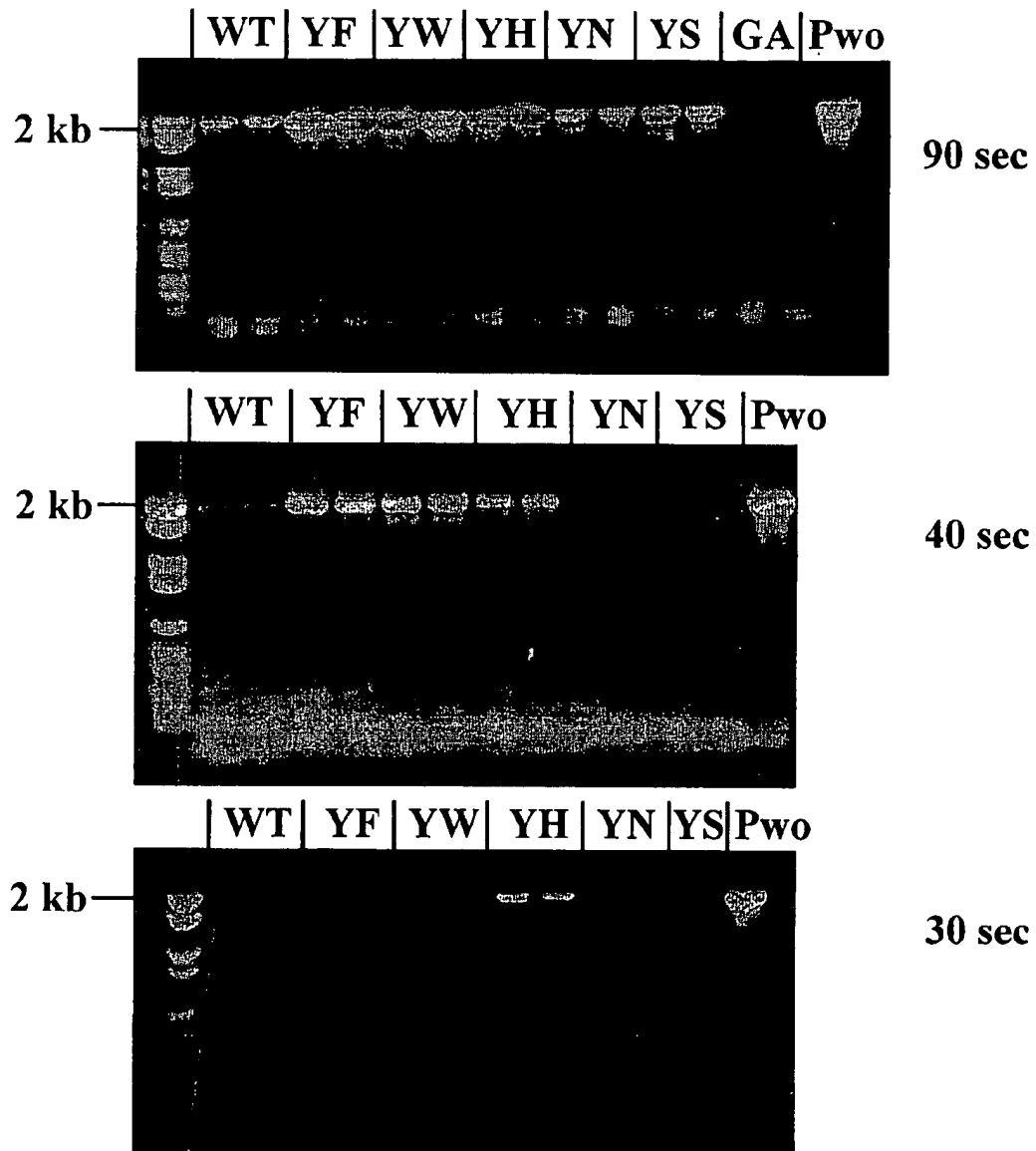


Figure 4

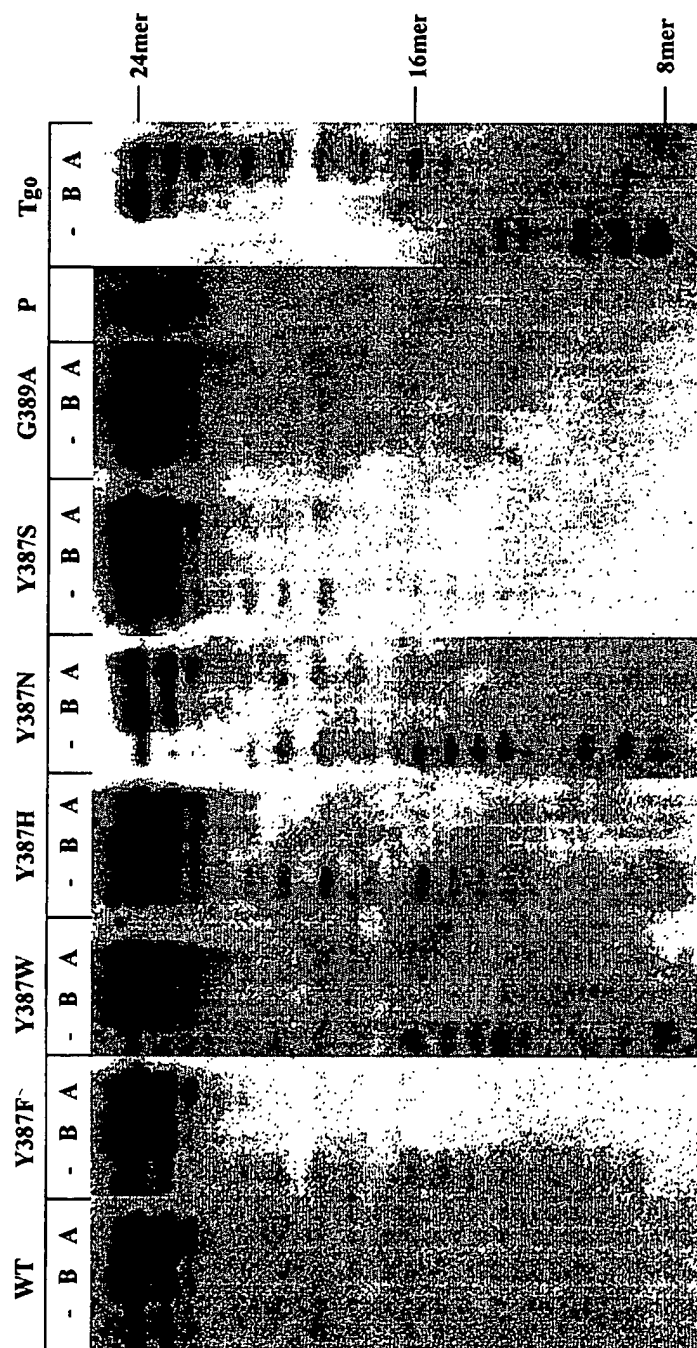


Figure 5

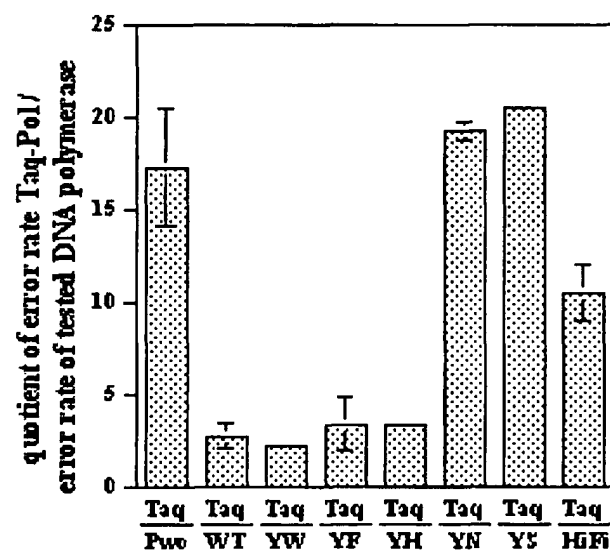


Figure 6

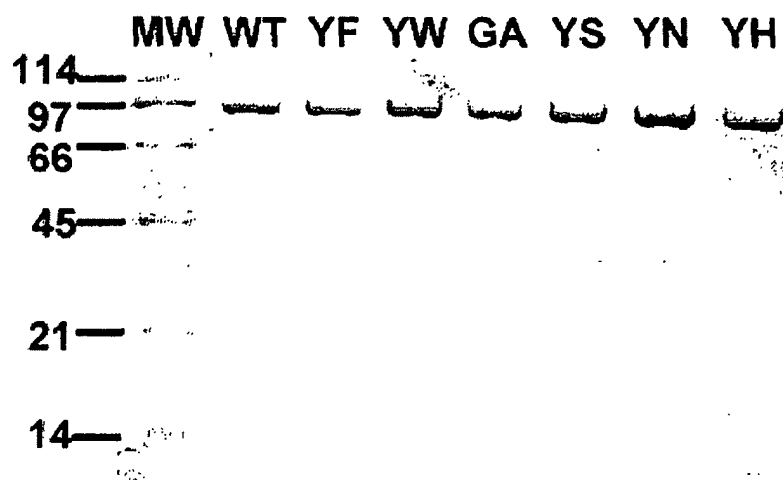
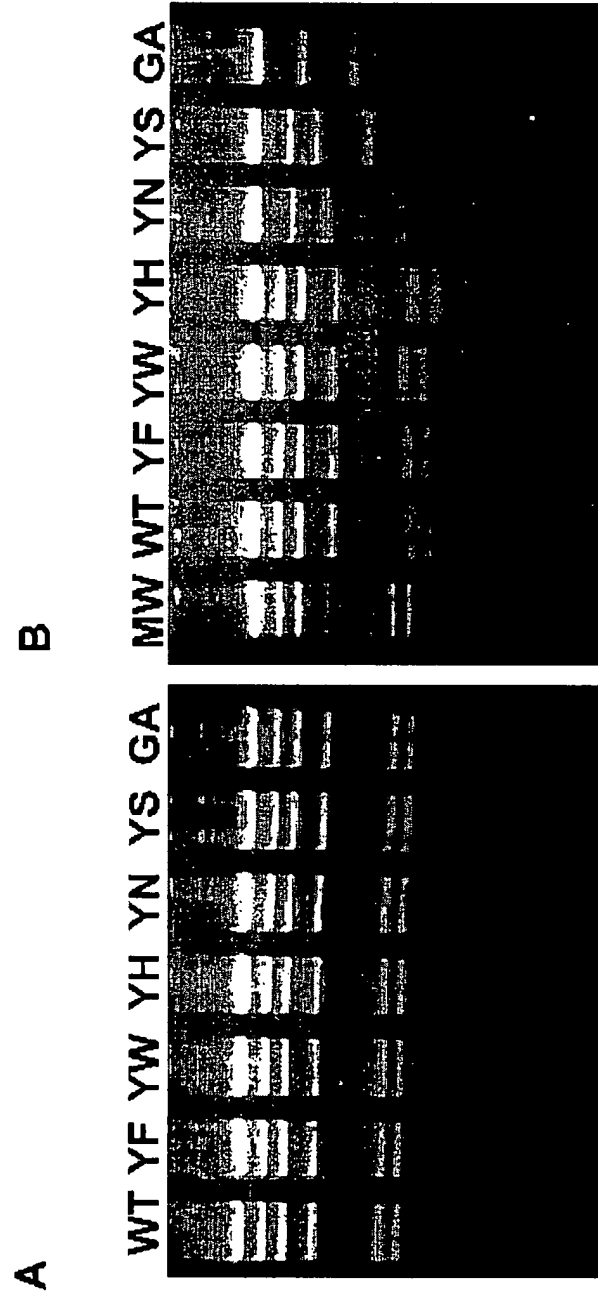


Figure 7



	E	R	R	R		G(Y)	K	E	E	L	W	E	consensus														
<i>T. aggregans</i>	E	Y	R	R	R	L	R	T	T	Y	L	G	G	Y	V	K	E	P	E	R	G	L	W	E	N		
<i>T. litoralis</i>	E	Y	K	R	R	R	L	R	T	T	Y	L	G	G	Y	V	K	E	P	E	R	G	L	W	E	N	
<i>T. tumidclans</i>	E	E	L	R	R	R	-	R	G	G	Y	A	G	G	Y	V	K	E	P	E	R	G	L	W	E	N	
<i>T. spec. 9N7</i>	E	E	L	R	R	R	-	R	G	G	Y	A	G	G	Y	V	K	E	P	E	R	G	L	W	E	N	
<i>T. gorgonarius</i>	E	E	L	R	R	R	-	R	E	S	Y	A	G	G	Y	V	K	E	P	E	R	G	L	W	E	N	
<i>P. spec. KOD</i>	E	E	L	R	R	R	-	R	E	S	Y	A	G	G	Y	V	K	E	P	E	R	G	L	W	E	N	
<i>P. abyssii</i>	E	E	Y	E	R	R	R	L	R	E	S	Y	A	G	G	Y	V	K	E	P	E	R	G	L	W	E	N
<i>P. furiosus</i>	E	E	Y	Q	R	R	R	L	R	E	S	Y	T	G	G	F	V	K	E	P	E	R	G	L	W	E	N
<i>P. horikoshii</i>	E	E	Y	E	R	R	R	L	R	E	S	Y	E	G	G	Y	V	K	E	P	E	R	G	L	W	E	N
<i>M. jannaschii</i>	E	Y	R	R	B	V	L	T	T	Y	E	G	G	Y	V	K	E	P	E	R	G	L	W	E	N		
<i>M. voltae</i>	S	Y	R	E	R	A	K	F	S	Y	E	G	G	Y	V	R	E	P	L	K	G	I	O	E	N		
<i>S. solfataricus</i>	T	S	A	L	I	K	G	K	G	Y	K	G	A	V	V	I	D	P	P	A	G	I	F	F	N		
<i>S. acidocaldarius</i>	T	A	A	V	I	K	G	K	K	Y	K	G	A	V	V	I	D	P	P	A	G	V	Y	F	F	N	
<i>P. islandicum</i>	T	K	A	I	I	K	G	K	K	Y	A	G	A	V	V	L	D	P	P	L	G	I	F	F	N		
<i>P. occultum</i>	S	E	A	L	I	K	G	K	K	Y	O	G	A	L	V	L	D	P	P	S	G	I	Y	F	F	N	
<i>A. pernix</i>	V	G	A	I	I	K	D	K	K	Y	R	G	A	I	V	L	D	P	P	V	G	I	F	F	N		
<i>S. chwalkuensis</i>	T	A	A	I	S	K	G	K	R	Y	K	G	A	V	V	I	D	P	P	A	G	V	F	F	N		

SEQ. ID. NO: 16
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 SEQ. ID. NO: 22
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 SEQ. ID. NO: 27
 SEQ. ID. NO: 28
 SEQ. ID. NO: 29
 SEQ. ID. NO: 30
 SEQ. ID. NO: 31
 SEQ. ID. NO: 32

Figure 8

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SEQ. ID. NO: 33

SEQ. ID. NO: 34

1/1

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 61/21
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 lys glu asn gly glu phe lys ile glu leu asp pro his phe gln pro tyr ile tyr ala
 121/41
 CTT CTC AAA GAT GAC TCC GCT ATT GAT GAA ATA AAA GCA ATA AAA GGC GAG AGA CAC GGA
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 181/61
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 241/81
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 301/101
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 361/121
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 421/141
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 481/161
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 ser tyr ala asp glu glu glu ala arg val ile thr trp lys asn ile asp leu pro tyr
 541/181
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 val asp val val ser asn glu arg glu met ile lys arg phe val gln ile val arg glu
 601/201
 AAA GAC CCG GAT GTC CTG ATA ACT TAC AAT GGA GAC AAC TTT GAT TTG CCG TAC CTT ATA
 lys asp pro asp val leu ile thr tyr asn gly asp asn phe asp leu pro tyr leu ile
 661/221
 AAA AGG GCA GAG AAG TTA GGA GTT ACT CTT CTC TTG GGG AGG GAC AAA GAA CAC CCC GAG
 lys arg ala glu lys leu gly val thr leu leu leu gly arg asp lys glu his pro glu
 721/241
 CCC AAG ATT CAC AGA ATG GGC GAT AGC TTT GCC GTG GAA ATT AAA GGC AGA ATT CAC TTT
 pro lys ile his arg met gly asp ser phe ala val glu ile lys gly arg ile his phe
 781/261
 GAT CTC TTC CCG GTT GTG CGG AGA ACC ATA AAC CTC CCA ACA TAC ACG CTT GAG GCA GTT
 asp leu phe pro val val arg arg thr ile asn leu pro thr tyr thr leu glu ala val
 841/281
 TAT GAA GCC GTC TTG GGA AAA ACC AAA AGC AAG CTG GGT GCG GAG GAA ATC GCC GCC ATC
 tyr glu ala val leu gly lys thr lys ser lys leu gly ala glu glu ile ala ala ile
 901/301
 TGG GAA ACA GAG GAG AGC ATG AAG AAG CTG GCC CAG TAC TCG ATG GAA GAT GCT AGG GCA
 trp glu thr glu glu ser met lys lys leu ala gln tyr ser met glu asp ala arg ala
 961/321
 ACT TAT GAA CTC GGA AAA GAG TTT TTC CCC ATG GAG GCA GAG CTA GCA AAG CTA ATA GGC
 thr tyr glu leu gly lys glu phe phe pro met glu ala glu leu ala lys leu ile gly
 1021/341
 CAA AGC GTA TGG GAC GTC TCA AGA TCA AGC ACT GGC AAC CTT GTA GAG TGG TAC CTG TTA
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 1081/361

Figure 9/2

SEQ. ID. NO: 33
 SEQ. ID. NO: 34

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 1141/381
 AGG CGT TTA AGG ACT ACT TAC CTG GGA GGA TAC GTA AAA GAG CCG GAA AGA GGC TTA TGG
 arg arg leu arg thr thr tyr leu gly gly tyr val lys glu pro glu arg gly leu trp
 1201/401
 GAG AAC ATC ACC TAT TTA GAC TTT AGG TGC CTA TAC CCC TCA ATT ATA GTT ACC CAC AAC
 glu asn ile thr tyr leu asp phe arg cys leu tyr pro ser ile ile val thr his asn
 1261/421
 GTC TCC CCT GAC ACT TTA GAA AGA GAA GGC TGC AAG AAT TAC GAT GTT GCC CCG ATA GTA
 val ser pro asp thr leu glu arg glu gly cys lys asn tyr asp val ala pro ile val
 1321/441
 GGT TAT AAG TTC TGC AAG GAT TTT CCC GGT TTC ATT CCA TCT ATA CTC GGG GAA TTA ATC
 gly tyr lys phe cys lys asp phe pro gly phe ile pro ser ile leu gly glu leu ile
 1381/461
 ACA ATG AGG CAA GAA ATA AAG AAG AAG ATG AAA GCT ACA ATT GAC CCA ATA GAA AAG AAA
 thr met arg gln glu ile lys lys lys met lys ala thr ile asp pro ile glu lys lys
 1441/481
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 met leu asp tyr arg gln arg ala val lys leu his ala asn ser tyr tyr gly tyr met
 1501/501
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 gly tyr pro lys ala arg trp tyr ser lys glu cys ala glu ser val thr ala trp gly
 1561/521
 AGG CAC TAC ATA GAA ATG ACC ATA AAA GAG ATA GAG GAG AAA TTT GGA TTT AAG GTG CTA
 arg his tyr ile glu met thr ile lys glu ile glu glu lys phe gly phe lys val leu
 1621/541
 TAT GCC GAC ACT GAT GGT TTT TAC GCC ACA ATA CCG GGA GAA AAA CCT GAA ACA ATC AAA
 tyr ala asp thr asp gly phe tyr ala thr ile pro gly glu lys pro glu thr ile lys
 1681/561
 AAG AAA GCT AAG GAA TTC TTA AAA TAC ATA AAC TCC AAA CTT CCC GGT CTG CTC GAG CTT
 lys lys ala lys glu phe leu lys tyr ile asn ser lys leu pro gly leu leu glu leu
 1741/581
 GAG TAT GAG GGC TTT TAC TTG AGA GGA TTT TTC GTC GCA AAG AAG CGC TAT GCG GTT ATA
 glu tyr glu gly phe tyr leu arg gly phe phe val ala lys lys arg tyr ala val ile
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 asp glu glu gly arg ile thr thr arg gly leu glu val val arg arg asp trp ser glu
 1861/621
 ATA GCC AAA GAG ACC CAG GCT AAA GTC TTG GAG GCA ATA CTT AAA GAA GAT AGT GTC GAA
 ile ala lys glu thr gln ala lys val leu glu ala ile leu lys glu asp ser val glu
 1921/641
 AAA GCT GTG GAA ATC GTT AAG GAC GTT GTT GAG GAG ATA GCA AAA TAC CAA GTC CCG CTT
 lys ala val glu ile val lys asp val val glu glu ile ala lys tyr gin val pro leu
 1981/661
 GAA AAG CTT GTT ATC CAC GAG CAG ATT ACC AAG GAT CTA AGT GAA TAC AAA GCC ATT GGG
 glu lys leu val ile his glu gln ile thr lys asp leu ser glu tyr lys ala ile gly
 2041/681
 CCT CAT GTA GCA ATA GCA AAG AGG CTT GCT GCA AAG GGA ATA AAA GTG AGA CCC GGC ACG
 pro his val ala ile ala lys arg leu ala ala lys gly ile lys val arg pro gly thr
 2101/701
 ATA ATA AGC TAT ATC GTC CTC AGG GGA AGC GGA AAG ATA AGT GAC AGG GTA ATT TTG CTT
 ile ile ser tyr ile val leu arg gly ser gly lys ile ser asp arg val ile leu leu
 2161/721

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SEQ. ID. NO: 33

SEQ. ID. NO: 34

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 2221/741
 CTG CCG GCG GTG CTT AGG ATC CTT GAA GCC TTC GGC TAC AGA AAA GAG GAC TTA AAA TAC
 leu pro ala val leu arg ile leu glu ala phe gly tyr arg lys glu asp leu lys tyr
 2281/761
 CAA AGC TCA AAA CAG GTT GGA CTG GAC GCG TGG CTT AAG AAG TAG
 gln ser ser lys gln val gly leu asp ala trp leu lys lys AMB



European Patent
Office

EUROPEAN SEARCH REPORT

Application Number
EP 01 10 4583

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
D,X	PISANI, F.M. ET AL.: "Amino Acid Residues Involved in Determining the Processivity of the 3'-5' Exonuclease Activity in a Family B DNA Polymerase from the Thermoacidophilic Archaeon Sulfolobus solfataricus" BIOCHEMISTRY, vol. 37, no. 42, 20 October 1998 (1998-10-20), pages 15005-15012, XP002145197	1-5,9-13	C12N15/52 C12N9/00
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The present search report has been drawn up for all claims			
Place of search BERLIN		Date of completion of the search 11 July 2001	Examiner Fuchs, U
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date O : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document</p>			

EPO FORM 1503 (01/02) (P04001)



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Application Number
EP 01 10 4583

DOCUMENTS CONSIDERED TO BE RELEVANT			
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P,X	<p>BÖHLKE, K. ET AL.: "PCR performance of the B-type DNA polymerase from the thermophilic euryarchaeon Thermococcus aggregans improved by mutations in the Y-GG/A motif"</p> <p>NUCLEIC ACIDS RESEARCH, vol. 28, no. 20, 15 October 2000 (2000-10-15), pages 3910-3917, XP002171733</p> <p>* the whole document *</p> <p>-----</p>	1-14	
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